disease. Over 70 genotypes of HPV have been identified since the recognitionof HPV as the main etiological factor for cervical cancer.

Certain HPV genotypes were selectively found in the lesions of specific location or progression stage, which rendered the biological diversity of HPV infection realized. Among the HPV genotypes detected in the anogenital area, over 10 genotypes have been classified as the high-risk group that are associated with an elevated risk for developing cervical cancer. Based on these findings, characterization of the biological differences of HPV infection is considered to be of significant importance to the diagnosis and prevention of cervical cancer.

For the diagnosis of cervical cancer at its early stage, Pap smear test has been most commonly used which is a cytological test performed as follows : old cells removed from the outermost layer of cells from the surface of thecervix are stained and examined forhistopathological characteristics of HPV infection including koilocytosis, formation ofperinuclear halo in the epithelial cells.

However, due to the low diagnostic efficiency(1-15%) of Pap test together with other limitations, additional methods such as colposcopy are necessary for more dependable diagnosis. Colposcopic screening can detect HPV infection up to70% but has disadvantages including high cost of the equipment, the need for skilled interpreters, and incapability of determining HPV genotypes to distinguish between the high-risk and low-risk infection.

Therefore, efforts have been made continuously to develop techniques for the detection of HPV and identification of HPV genotypes to supplement conventional screening methods for cervical cancer and its precursors including Pap test.

The methods for detection of HPV and identification of HPV genotypes can be classified into two groups, i. e., direct detection of HPV DNA and detection of amplified HPV DNA. The methods for direct detection of HPV DNA include liquid hybridization (Hybrid Capture kit by Digene Diagnostics, Silver Spring, MD, USA, www. digene. com), Sourthern blot and dot blot with HPV type-specific probes, filter in situ hybridization (FISH) and the like, and the methods for the detection of amplified DNA include typespecific PCR (polymerase chain reaction) and

general-primer PCR. In particular, genotype analyses of amplified HPV DNA by general primer sets are commonly performed by employing dot blot hybridization, microtiter plate hybridization, or line probe assay. Among these methods, liquid hybridization by Hybrid Capture and line probe assay following general-primer PCR have been considered most suitable for diagnostic purposes. The line probe assay can detect about 20 different HPV genotypes by immobilized oligonucleotide probes on a nitrocellulose membrane, however, it lacks reliability due to low sensitivity and difficulties in data interpretation. Commercialized Hybrid Capture kit can detect HPV DNA in clinical samples without PCR amplification and distinguish between high-risk and low-risk HPV groups. However, the fact that Hybrid Capture kit cannot identify the genotypes of infecting HPV limits accurate risk determination since the risk factor amongst the high-risk HPV is not the same, in other words, intermediate-risk types are included in the high-risk group.

Moreover, the use of RNA probe may pose low stability of the kit, and also possibility of contamination cannot be excluded.

Under these circumstances, there have been strong reasons for exploring and developing a simple and accurate method for detection of HPV infection and identification of the genotype of infecting HPV.

[Summary of the Invention]

A primary objective of the present invention is, therefore, to provide a genotyping kit for diagnosis of HPV infection.

The other objective of the invention is to provide a process for preparing the HPV genotyping kit which is stable and has low contamination.

Another objective of the invention is to provide a process for preparing the HPV genotyping kit which can diagnose existence and genotype of HPV in a simple and accurate manner and in large quantities.

[Brief Description of the Drawings]

Figure 1 is a scheme showing the steps of preparing oligonucleotide microarray of HPV according to the present invention.

Figure 2 is a scheme fixing to slide HPV oligonucleotide according to the present invention.

Figures 3a and 3b are schemes fixing to silylated slide HPV oligonucleotide according to the present invention.

Figure 4 is a result of microarray hybridization using HPV DNA type 16 according to the present invention.

Figure 5 is a result of microarray hybridization using HPV DNA type 18 according to the present invention.

Figure 6 is a result of microarray hybridization using DNA isolated from clinical samples.

[DETAILED DESCRIPTION OF THE INVENTION]

The genotyping kit of the invention for diagnosis of human papillomavirus (HPV) infection comprises : a DNA oligonucleotide chip and means for labeling sample hybridized with the said DNA chip.

According to the invention, the genotyping kit is very advantageous to predict risk degree of infection since it diagnoses existence and genotype of HPV in a simple and accurate manner.

And, the genotyping kit is stable and shows low contamination because of using DNA chip instead of RNA chip.

The genotyping kit of the invention comprises microarray prepared by affixing via Schiff's base reaction of 5'terminal amine-linked DNA probes that have nucleotide sequences complementary to the DNA of HPV and aldehyde-derivatized surface of glass and biotin-binding protein to detect hybridization of biotin-labeling HPV DNA amplified product and the probe oligomer affixing to microarray.

The DNA probe is L1 DNA of HPV.

The kit can easily diagnose HPV infection, and can exactly determine the genotype of the HPV.

The process for preparing DNA chip of the invention comprises: step of preparing 5'terminal amine-linked DNA probes that have nucleotide sequences complementary to the DNA of HPV(step 1); step of preparing an aldehyde-derivatized surface of a solid support, preferably glass(step 2); and affixing DNA probes prepared in Step 2 to the glass via Schiff's base reaction(step 3).

HPV genotyping kit of the invention is an implement that can detect HPV infection in a simple

and accurate manner, as well as identify the types of infecting HPV, therefore, it may contribute to early diagnosis, prevention and treatment of cervical cancer.

The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

1. Preparation of oligonucleotide microarray

As you can see from Figure 1, oligonucleotide microarray was prepared by synthesizing HPV oligonucleotide(step 10) and affixing the HPV oligonucleotide to slide(step 11).

(1) synthesis of HPV oligonucleotide

Genotype-specific probe for each HPV type possessing amine group at 5'terminal of the sequence was prepared for the detection of HPV genotypes.

The nucleotide sequence of each probe is as follows :

[Table 1] Oligonucleotide sequences

Name	Sequence	Description
HPV 16	5'-Amino-gtcattatgtgctgccatatctacttcaga-3'	Probe
HPV 18	5'- Amino-tgcttctacacagtctcctgtacctgggca-3'	Probe
HPV 31	5'- Amino-tgtttgtgctgcaattgcaaacagtgatac-3'	Probe
HPV 33	5'- Amino-tttatgcacacaagtaactagtgacagtac-3'	Probe
HPV 35	5'- Amino-gtctgtgtgttctgctgtgtcttctagtga-3'	Probe
HPV 45	5'- Amino-acacaaaatcctgtgccaagtacatatgac-3'	Probe
HPV 51	5'- Amino-agcactgccactgctgcggtttccccaaca-3'	Probe
HPV 52	5'- Amino-tgctgaggttaaaaaggaaagcacatataa-3'	Probe
HPV 56	5'- Amino-gtactgctacagaacagttaagtaaatatg-3'	Probe
HPV 6	5'- Amino-atccgtaactacatcttccacatacaccaa-3'	Probe
HPV 11	5'- Amino-atctgtgtctaaatctgctacatacactaa-3'	Probe
HPV 58	5'- Amino-attatgcactgaagtaactaaggaaggtac-3'	Probe

(2) Affixing HPV oligonucleotide(Fig 2)

Oligonucleotide probes(Table 1) synthesized on Aldehyde-derivated silylated slide(Fig 3a)

was fixed as follows :

each probe prepared above was dissolved in 3X SSC (0.05M sodium citrate, 0. 45MNaCl) at a concentration of 200pmol/㎛, and spotted onto an silylated slide (CSS100, CEL, Houston, TX, USA) to form an array of spots with size of 0.1μl(step 22) ; followed by performing Schiff's base reaction under an environment of 37℃ and over 70% humidity for 4 hours(step 24). The slide was washed with 0.2% (w/v) sodium dodecyl sulfate (SDS)(step 26), and with triple distilled water for 1 min(step 28). Then, the slide was treated with NaBH solution(0.1g NaBH, 30ml phosphate buffered saline (PBS), 10ml ethanol) for 5 minutes to reduce excessive aldehydes not reacted with amine(step 30), followed by washing with triple distilled water(step 32) and air-drying(step 34).

2. Preparation of samples and isolation of DNA

(1) Clinical Sample

Human cervical swabs was provided from Catholic University(Korea), detected whether it is infected as high-risk group using Hybrid Capture kit(Digene Diagnostics, MD, USA), and kept in DNA isolating buffer of Hybrid Capture kit. Before PCR amplification, DNA was concentrated and purified using iNtRON genomic DNA kit as followings:

(i) adding a proper quantity of proteinase K into sample of human cervical swabs and treating at 37℃ for 15hr, and then inactivating the enzyme at 95℃ for 10min.

(ii) adding a proper quantity of lysis buffer to pellet and incubating 65℃ for 10min.

(iii) adding and stirring a proper quantity of sol III and then leaving on the ice for 5min.

(iv) centrifuging at 1200 rpm for 5min and transferring supernatant into new tube.

(v) mixing a proper quantity of isopropanol and centrifuging at 12,000 rpm for 5min to collect pellet.

(vi) adding a proper quantity of 70% ethanol, centrifuging for 10min and dissolving collected pellet into buffer or distilled water.

(2) HPV cell line

Additionally, DNA extracted and purified from following cell lines were used as positive controls : SiHa cell line (HPV 16, KCLB 30035, Human squamous carcinoma, cervix) and HeLa cell line (HPV 18, KCLB 10002, Human epithelial carcinoma, cervix) which were purchased from Korean Cell Line Bank (Seoul National University, College of Medicine, Seoul, Korea). After

treating the cell lines as proteanase K and inactivating the enzyme at 95℃, HPV DNA was amplified using the part of them in PCR reaction.

3. HPV detection using oligonucleotide microarray

(1) PCR

Primer for detecting HPV infection used GP5+ and biotin linked GP6+(biotin-GP6+) and length of amplified sequence was 150bp. B-globin primer used to decide suitability of isolated DNA is PCO3/PCO4 and its length is 110 bp.

PCR was carried out by the following:

50μl of reaction mixture solution was prepared, and the solution comprises PCR buffer solution(50mM KCl, 4mM $MgCl_2$, 10mM Tris-HCl, pH 8.3), 0.1μg of DNA, 4.5mM $MgCl_2$, each 50pmol HPV primer GP5+(5'-TTTGTTACTGTGGTAGATACTAC-3'), biotin-GP6+(3'-biotin-CTTATACTAAATGTCAAATAAAAAG-5'), 40μM dATP(Pharmacia), 40μM dCTP(Pharmacia), 40μM dGTP(Pharmacia), 30μM dTTP(Pharmacia), 10μM biotin-dUTP(Boehringer Manheim), and 1 unit Taq polymerase(Takara, Japan).

DNA was amplified by 40 times repetition of steps of denaturating at 94℃ for 1min in PCR thermocycler(Perkin-Elmer Cetus, CA, USA), primer annealing at 40℃ for 1min, and extending at 72℃ for 1min.

2μl sample was taken from amplified PCR DNA solution, and mixed with loading dye. After electrophoresis at 2% agarose gel and dying into 1μg/ml of ethidium bromide(Sigma) for 15min, size of DNA was detected under ultraviolat rays. To judge DNA preservation of sample, B-globin gene amplification test was accomplished using PCO3/PCO4 primer set and the test used only amplified B-globin gene.

(2) Hybridization

Hybridization of HPV DNA amplifed by PCR was carried out at HPV oligonucleotide probe-fixed board. 200μl of cover slip(Grace Bio-Labs, USA) was used as hybridization reaction chamber.

20-30μl of amplified product was denatured at 95℃ for 10min, was leaved on the ice for 10min, and then reacted with probe affixed to silylated slide at 30-40℃ for 1-2hrs using as hybridization solution 6X saline-sodium phosphate-EDTA buffer(SSPE, Sigma, St. Louis, MO) and 0.2% sodium dodecyl sulfate(SDS, Sigma).

After hybridization reaction, glass was washed with 3X SSPE for 2min, 1X SSPE for 2min and

was dried at room temperature. After adding solution mixing 5μl of streptavidin-R-phycoerythrin conjugate(50 μg/ml) into 95μl of 3X SSPE and dying at room temperature for 25min, it was washed with 3X SSPE and dried, and then was analyzed using confocal laser scanner(GMS 418 Array Scanner, Takara)(Excitation 480nm, emission>520nm).

Since DNA chip can analyze gene mutation in a short time in large quantities, importance of it is increased. The invention relates to a genotyping kit for diagnosing patients infected with human papillomavirus (HPV), and a method for preparing the said kit.

In Example, single stranded HPV type-specific probe(approximately 30mer) was affixed into microscopic slide glass, and was hybridized with HPV target DNA amplified from HPV cell line or plasmid. Affixture of probe was carried out using Schiff base reaction between aldehyde of slide glass and amine of probe and the produced imine bond was reduced by $NaBH_4$ to increase chemical stability(Fig 3b).

Meanwhile, an Example prepared microarray using 10 high-risk group probes such as HPV types 16, 18, 31, 33, 35, 45, 51, 52, 56, 58, and the efficiency of hybridization was tested by changing concentration of probe DNA solution from 100pmol/μl to 1nmol/μl to calculate most desirable concentration of probe. The result showed the best reaction efficiency at 200pmol/μl.

Microarray affixing complementary probes to 10 HPV genotypes prepared at most suitable conditions was tested using PCR product of each genotype plasmids. PCR of HPV 16 and HPV 18 cell lines used GP5+/biotin-GP6+ primer set and biotin-dUTP. The result is that biotin-labeled 150bp amplifier was obtained in high yield.

After denaturing the amplifier, and hybridizing it with microarray at 30-40℃ for 1-2hrs using hybridization chamber, hybridization site was dyed by streptavidin-R-phycoerythrin including biotin-binding protein. Streptavidin-R-phycoerythrin is conjugate of protein having 4 biotin-binding site and fluorophore which is high extinction coefficient and when scanning microarray using confocal laser scanner, hybridization site can be detected in high sensitivity.

Therefore, adding biotin-dUTP and labeling target DNA optionally in PCR reaction are necessary for increase of detection sensitivity. Hybridization signal of labeled target DNA was superior to using only biotinylated primer.

Figures 4 and 5 show the result of microarray hybridization using HPV 16 and HPV 31 DNAs amplified from cell lines. In figures 4 and 5, circles under figures mean location according to a

kind of probe, and PC(positive control) means a maker for showing site of probes.

In figures 4 and 5, hybridization signal coming from amplified products of HPV 16 and HPV 18 DNAs showed only at each HPV 16 and HPV 18 clearly without serious cross-hybridization.

Figure 6 shows the result of microarray hybridization using HPV DNA amplified from clinical sample. The sample was detected as high-risk group using Hybrid Capture kit(Digene Diagnostics, MD, USA), and sample of the left side of figure 6 was HPV 16 and sample of the right side of figure 6 was HPV 58. Under the same test condition, HPV 16, 31, 51, 56 and 58 was detected as high-risk group using Hybrid Capture kit.

The Example of the present prepared HPV genotyping kit using 10 probes affixed oligonucleotide microarray, and to detect more broadly, the invention can use HPV DNA chip which a kind of probe oligomer is increased.

HPV genotyping kit using oligonucleotide microarray according to the invention is very advantageous to diagnose existence and genotype of HPV in a simple and accurate manner and in large quantities.

[Advantageous Effect]

HPV genotyping kit according to the present invention can diagnose existence and genotype of HPV in a simple and accurate manner and in large quantities.

The invention can increase stability and prevent contamination.

The invention can detect genotype in a simple and accurate manner by analyzing hybridization signal using laser scanner, since it uses fluorescent material as means for labeling .

What is claimed is :

1. A Human Papillomavirus (HPV) genotyping kit which comprises : (i) a DNA chip and (ii) means for labeling sample DNA hybridized with the said DNA chip.

2. The HPV genotyping kit of claim 1 wherein the DNA chip is formed via Schiff's base reaction of 5'terminal amine-linked DNA probes that have nucleotide sequences complementary to the DNA of HPV and aldehyde-derivatized surface of glass.

3. The HPV genotyping kit of claim 2 wherein the DNA probe is L1 DNA of HPV.

4. The HPV genotyping kit of claim 2 or claim 3 wherein concentration of the DNA probe is 150-250 pmol/μl.

5. The HPV genotyping kit of claim 1 or claim 2, further comprising the means for amplifying the sample DNA.

6. The HPV genotyping kit of claim 5 wherein the means for amplifying the sample DNA comprises GP5+/6+ primer system and 150 bp amplifier.

7. The HPV genotyping kit of claim 6 wherein the means for amplifying the sample DNA further comprises biotin-dUTP.

8. The HPV genotyping kit of claim 6 or claim 7 wherein the means for labeling is a biotin-binding protein.

9. The HPV genotyping kit of claim 8 wherein the biotin-binding protein is streptavidin-R-phycoerythrin.

10. The HPV genotyping kit of claim 1 or claim 2 wherein the DNA chip further comprises position markers to locate DNA probes.

11. A process for preparing a HPV genotyping kit comprising the steps of :

(i) step of preparing 5'terminal amine-linked DNA probes that have nucleotide sequences complementary to the DNA of HPV(step 1);

(ii) step of preparing an aldehyde-derivatized surface of a solid support, preferably glass(step 2); and

(iii) affixing DNA probes prepared in Step 1 to the glass via Schiff's base reaction(step 3).

12. The process for preparing a HPV genotyping kit of claim 11 wherein the DNA probe is L1 DNA of HPV.

13. The process for preparing a HPV genotyping kit of claim 11 or claim 12 wherein concentration of the DNA probe is 150-250 pmol/μl.

14. The process for preparing a HPV genotyping kit of claim 13 wherein concentration of the DNA probe is 200 pmol/μl.

15. The process for preparing a HPV genotyping kit of claim 11 or claim 12, further comprising the reduction of aldehyde performed by the aid of a reducing agent, $NaBH_4$.